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Comprehensive sequence analysis of the E1A proteins of human and simian adenoviruses

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Abstract

Despite extensive study of human adenovirus type 5 E1A, surprisingly little is known about the E1A proteins of other adenoviruses. We report here a comprehensive analysis of the sequences of 34 E1A proteins. These represent all six primate adenovirus subgroups and include all human representatives of subgroups A, C, E, and F, eight from subgroup B, nine from subgroup D, and seven simian adenovirus E1A sequences. We observed that many, but not all, functional domains identified in human adenovirus type 5 E1A are recognizably present in the other E1A proteins. Importantly, we identified highly conserved sequences without known activities or binding partners, suggesting that previously unrecognized determinants of E1A function remain to be uncovered. Overall, our analysis forms a solid foundation for future study of the activities and features of the E1A proteins of different serotypes and identifies new avenues for investigating E1A function.

Introduction

To date, 51 human adenovirus (HAdV) serotypes have been isolated and these have been divided into six subgroups, termed A–F, according to a variety of criteria (Benkö et al., 2000). In primate adenoviruses, Early Region 1A (*E1A*) is the first gene expressed after infection of the host cell (Nevins et al., 1979). It has been most extensively characterized in subgroup C HAdV type five (HAdV-5) and HAdV-2, which are virtually identical in sequence and will be treated as interchangeable in this report. In these viruses, *E1A* encodes two major proteins of 289 and 243 amino acid residues (R), which differ only by the presence of 46 additional amino acids in the larger protein as a result of differential splicing of the primary RNA transcript (Perricaudet et al., 1979). The E1A proteins are localized in roughly equal amounts in both the cytoplasm and nucleus (Rowe et al., 1983; Turnell et al., 2000). Three additional mRNA species are produced at later times that encode, or are predicted to encode, E1A proteins of 217, 171, and 55R (Stephens and Harlow, 1987; Ulfendahl et al., 1987). The E1A proteins of HAdV-12 from subgroup A have been studied less extensively, but appear at least superficially similar to those of HAdV-5. For example, the *E1A* gene of HAdV-12 encodes two major proteins of 266 and 235R that share homology with the HAdV-5 289 and 243R E1A proteins (Perricaudet et al., 1980), as well as several minor species (Sawada and Fujinaga, 1980; Brockmann et al., 1990). The E1A proteins of HAdV-5 and HAdV-12 appear more or less functionally equivalent as they activate expression of other viral early genes and reprogram cell growth to provide an optimal environment for viral replication (Bayley and Mymryk, 1994; Boulanger and Blair, 1991; Dyson and Harlow, 1992; Frisch and Mymryk, 2002; Gallimore and Turnell, 2001; Moran, 1994; Peeper

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and Zantema, 1993; Shenk and Flint, 1991). These functions make them both essential for a productive infection by their respective viruses (Byrd et al., 1988; Jones and Shenk, 1979).

HAdV-5 and HAdV-12 E1A proteins interact with a variety of cellular proteins, including transcriptional co-activators such as the CREB-binding protein (CBP) and p300 (Arany et al., 1995; Dorsman et al., 1997; Eckner et al., 1994; Jelinek and Graham, 1992; Lundblad et al., 1995; Sawada et al., 1997; Wang et al., 1993b), the p300/CBP Associated Factor (pCAF) (Reid et al., 1998; Shuen et al., 2003), and the transcriptional co-repressors CtBP (Grand et al., 1998; Schaeper et al., 1995) and BS69 (Ansieau and Leutz, 2002; Hateboer et al., 1995). Similarly, E1A proteins from both viruses interact with the TATA-binding protein (TBP) component of the general transcriptional machinery (Boyer and Berk, 1993; Geisberg et al., 1994; Grand et al., 1998; Hateboer et al., 1993; Horikoshi et al., 1991; Song et al., 1995). In addition, both HAdV-5 and HAdV-12 E1A proteins directly target the retinoblastoma tumor suppressor gene product (Rb) and related family members p130 and p107 (Egan et al., 1989; Ewen et al., 1991; Hannon et al., 1993; Jelinek and Graham, 1992; Peeper et al., 1992; Whyte et al., 1988), and the cyclin-dependent kinase inhibitor p27 (Mal et al., 1996b; Nomura et al., 1998) that regulates the cell division cycle. HAdV-5 and HAdV-12 also bind the SUMO conjugase UBC9 (Hateboer et al., 1996) and proteasome components (Grand et al., 1999; Turnell et al., 2000). Because of these many interactions with cellular regulatory proteins, the multifunctional E1A proteins influence a wide variety of transcriptional and cell cycle events (Bayley and Mymryk, 1994; Dyson and Harlow, 1992; Gallimore and Turnell, 2001; Moran, 1994; Peeper and Zantema, 1993; Shenk and Flint, 1991). Importantly, the *E1A* genes of many different HAdVs, including HAdV-5 and HAdV-12 function as oncogenes in cultured rodent cells (Bayley and Mymryk, 1994; Endter and Dobner, 2004; Gallimore and Turnell, 2001). However, the ability of these transformed cells to induce tumors in animals varies greatly depending on the species of E1A used (Endter and Dobner, 2004; Gallimore and Turnell, 2001; Huebner et al., 1962; Trentin et al., 1962; Williams et al., 1995). Intriguingly, HAdV-5 *E1A*, which is classified as non-oncogenic in rodent systems, has been shown to function as a tumor suppressor gene by inhibiting tumorigenesis and metastasis (Frisch and Mymryk, 2002; Mymryk, 1996) and may have some utility in cancer therapy (Ueno et al., 2001).

Despite the large number of studies focusing on HAdV-5 E1A, relatively little is known about the function of the E1A proteins of other adenoviruses, including HAdV-12. This raises questions regarding how representative HAdV-5 E1A is of the other E1A proteins. It seems unlikely that each of the different proteins performs identical functions. For example, in addition to significant differences between the tumorigenic effects of HAdV-5 and HAdV-12 E1A pointed out above, HAdV-12 E1A but not HAdV-5 E1A represses major histocompatibility complex class I transcription in the infected cell (Ackrill and Blair, 1988; Bernards et al., 1983; Friedman and Ricciardi, 1988). The two proteins have differential effects on expression of the junB and junD genes (de Groot et al., 1991), and the N-terminus of HAdV-12 E1A harbors a trans-activation function not detectable in HAdV-5 E1A (Lipinski et al., 1997). Despite these known differences, and potentially many more yet to be identified, the E1 regions of subgroup A, B, C, and E viruses can complement an E1-defective HAdV-5 virus for growth in culture, suggesting that at least key functions are conserved (Rademaker et al., 2002).

Previous comparisons of E1A sequences from human and simian adenoviruses (SAdVs) identified four regions of high sequence homology designated conserved regions (CR) 1, 2, 3, and 4 (Avvakumov et al., 2002b; Kimelman et al., 1985). As expected from their strong conservation, these regions are critical for many activities of E1A (Bayley and Mymryk, 1994; Frisch and Mymryk, 2002; Gallimore and Turnell, 2001).

We report here a comprehensive comparison of the sequences of 34 E1A proteins. Many functional domains identified in human adenovirus type 5 E1A are recognizably conserved among the other E1A proteins. However, we identify highly conserved sequences without known activities or binding partners, providing new avenues to discover novel E1A targets and functions. Our analysis also forms a solid foundation from which study of the activities and features of other E1A proteins can begin.

Results and discussion

We report here the sequences of the *E1A* genes of HAdV types 1, 6, 8, 14, 16, 18, 23, 28, and 31. These were determined as described previously (Avvakumov et al., 2002b), and their GenBank accession numbers are listed in Table 1. Combining these with the newly available E1A sequences from HAdV-11, 26, 35, 36, 49, and 51 and SAdV-21 through 24, we report here a comprehensive comparison of the predicted sequences of thirty-four E1A proteins. These include all human representatives of subgroups A, C, E, and F, eight from subgroup B and nine from subgroup D, as well as seven SAdV E1A sequences. These proteins range in size from 249 to 289 residues, with the subgroup C proteins being the largest and the subgroup D and F proteins the smallest.

The organization of the E1A sequences into six subgroups is displayed as an unrooted phylogenetic tree (Fig. 1). The most commonly accepted source of phylogenetic information for the AdV is the hexon sequence and our subgroup assignations agree with current designations for those AdV with known hexon sequence (Benkö et al., 2000). It is also clear from our analysis that there is little, if any, distinction between most of the currently known human and simian AdV E1A sequences. Indeed, virus neutraliza-

Table 1
Genbank accession numbers, subgroup classification and size of the largest E1A protein products of human and simian adenoviruses

Virus	Accession no.	Subgroup	E1A size (aa)[a]	Source[b]
HAdV-1	AAS65966	C	289	LUMC-MCB[c]
HAdV-2	P03254	C	289	–
HAdV-3	AAM46821	B:1	261	–
HAdV-4	P10407	E	257	–
HAdV-5	P03255	C	289	–
HAdV-6	AAS65967	C	289	CDC[d]
HAdV-7i	P03256	B:1	261	–
HAdV-7ii	AAA42455	B:1	261	–
HAdV-8	AAS65968	D	253	ATCC[e]
HAdV-9	AAD16301	D	251	–
HAdV-11	AAN62486	B:2	262	–
HAdV-12	P03259	A	266	–
HAdV-14	AAS65969	B:2	261	ATCC
HAdV-16	AAS65970	B:1	261	ATCC
HAdV-17	AF108105	D	253	–
HAdV-18	AAS65971	A	278	ATCC
HAdV-21	AAM46822	B:1	261	–
HAdV-23	AAS65972	D	253	ATCC
HAdV-26	AY678272	D	251	Crucell[f]
HAdV-28	AAS65973	D	253	ATCC
HAdV-31	AAS65974	A	265	CDC
HAdV-35	AX049983	B:2	262	–
HAdV-36	AAQ72377	D	253	–
HAdV-40	P10541	F	249	–
HAdV-41	P10542	F	251	–
HAdV-49	AY678273	D	253	Crucell
HAdV-51	AY678274	D	253	Crucell
SAdV-7i	P06499	A	266	–
SAdV-7ii	CAA25511	A	266	–
SAdV-21	DAA00791	B	262	–
SAdV-22	AAS10355	E	258	–
SAdV-23	AAS10391	E	258	–
SAdV-24	AAS10427	E	258	–
SAdV-25	AAL35510	E	257	–

[a] aa, amino acids.
[b] Listed only for newly determined sequences.
[c] Leiden University Medical Centre, Department of Molecular Cell Biology virus collection.
[d] Centers for disease control and prevention.
[e] American Type Culture Collection.
[f] Sequence obtained from Crucell, Leiden, The Netherlands before Genbank submission.

tion by specific antisera has previously indicated that SAdV are related to HAdV (Wigand et al., 1989; Willimzik et al., 1981). Of the sequences analyzed here, those within subgroup C are the most closely related. This suggests that these viruses evolved together to infect a specific subset of target cells and that genetic divergence is not well tolerated. Subgroup B is clearly composed of two distinct subsets, which corresponds well with their phylogenetic classification as B:1 and B:2 (Benkö et al., 2000). Interestingly, SAdV-21 E1A is intermediate between these two subsets. Subgroup D contains two viruses-HAdV-23 and HAdV-51-whose E1A protein, though not nucleotide, sequences are identical. Of note, the SAdV-7 sequences are highly divergent from the subgroup A HAdV sequences, suggesting that they could be classified as a distinct subset, as previously done with the subgroup B viruses, or possibly even be considered to represent a seventh subgroup. However, alternative classification of SAdV-7 must await the determination of its hexon sequence.

We noticed a duplication of sequence in HAdV-18 E1A between CR1 and CR2 (Fig. 2). The duplicate sequences are ATSEGLLLTEPPVISPV and AASDGLLLTDPPILSPV, which are clearly recognizable despite evident divergence. This duplication was observed in HAdV-18 obtained from the ATCC and CDC, and nearly doubles the distance between CR1 and CR2 in this protein. A short duplication of the residues QPE is also apparent in the subgroup C E1A proteins in the same general region (Fig. 2).

Refining the conserved regions of E1A

Alignment of the 34 E1A sequences (Figs. 2 and 3) did not alter the boundaries of CR1 and CR3, which remain at alignment position (a.p.) 50–81 and 199–246, respectively. Within CR3, the four cysteine residues that form the zinc finger (Culp et al., 1988) are absolutely conserved at alignment positions 209, 212, 226, and 229. The boundaries

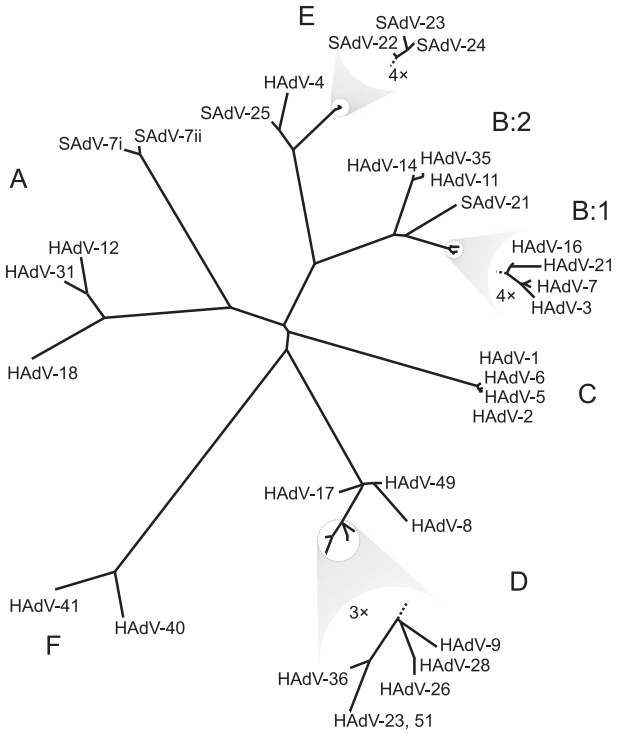

Fig. 1. Phylogenetic tree for the adenovirus E1A proteins. An unrooted tree was generated for the E1A proteins based on sequence alignment with the PHYLIP and TreeView programs as described in Materials and methods. Branch length is proportional to genetic distance. Each species of E1A is labeled at the tip of its representative branch. The adenoviral subgroups are labeled as A–F according to convention. Some regions are enlarged by the indicated magnification factor for better visualization.

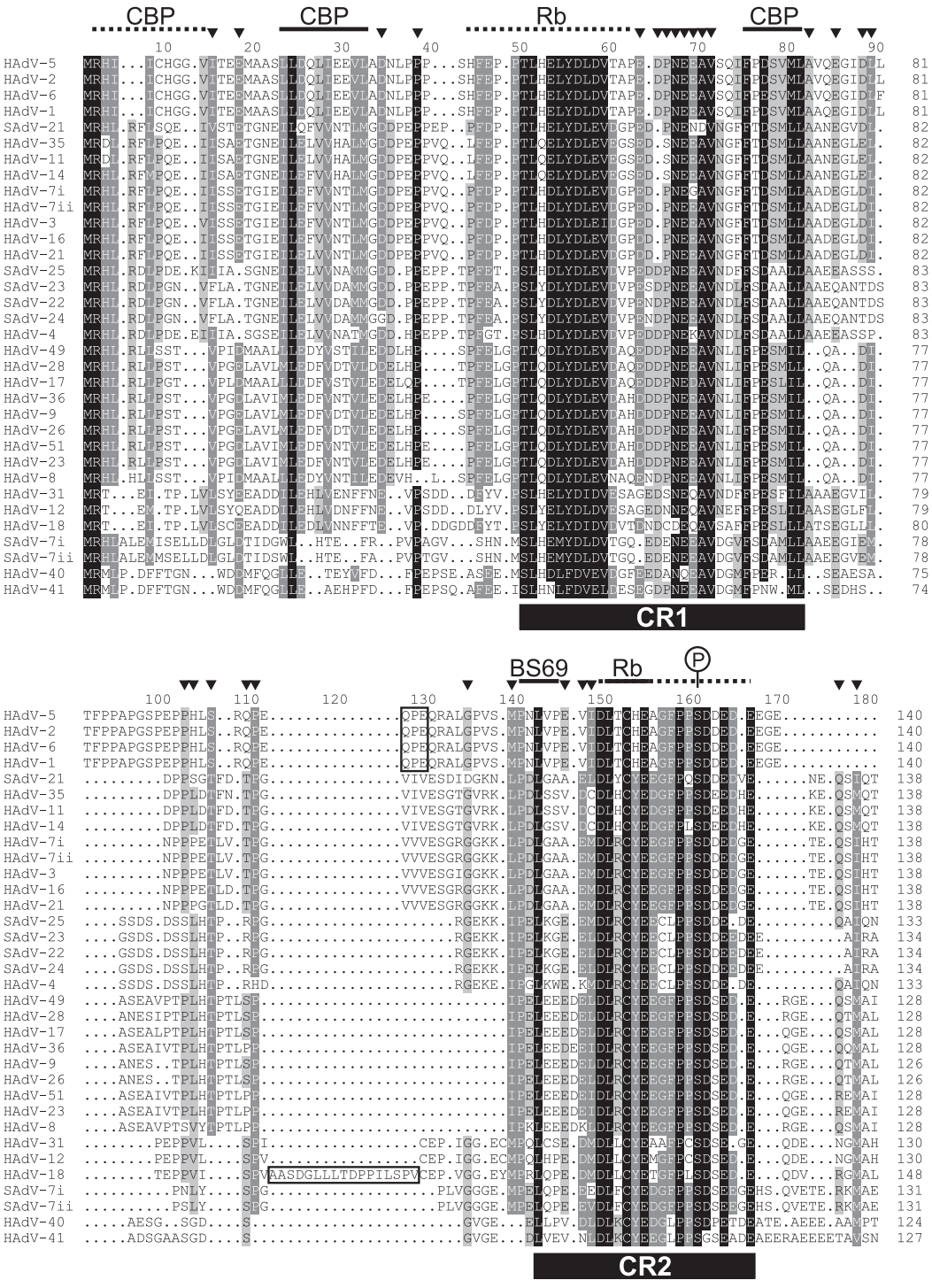

Fig. 2. Alignment of the N-terminal sequences of adenovirus E1A proteins. Sequences were aligned as described in Materials and methods and shaded for conservation. Darker shading corresponds to higher levels of conservation. Gaps are indicated as dots. Arrowheads indicate highly conserved residues (>60% similarity) without known function. The positions of the conserved regions (CR) are indicated as black bars. Numbers at the end of each row indicate the last residue's position within the particular sequence. The binding sites for CBP, Rb and BS69, as well as the conserved phosphorylation site are indicated.

of CR2 shift slightly to a.p. 142 to 166. However, incorporation of the additional 19 sequences into the current analysis has allowed us to identify substantially greater conservation within the C-terminus of the E1A proteins. This expands CR4 to encompass a.p. 296–353, with an overall relative similarity between the different E1A proteins of 60%. CR4 is clearly composed of two discrete regions of higher conservation, with an intervening less conserved sequence that is generally acidic and often glycine-rich.

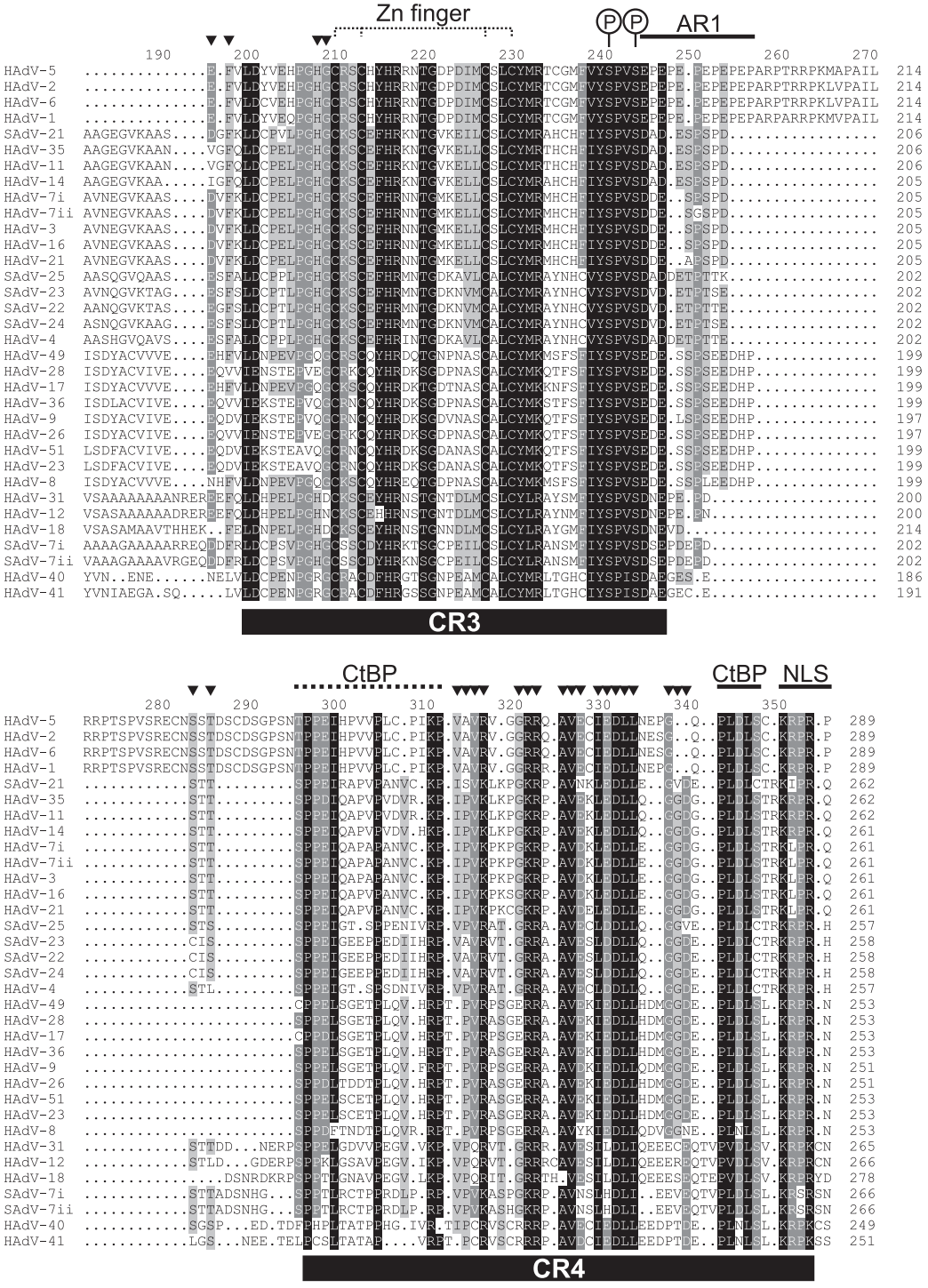

Fig. 3. Alignment of the C-terminal sequences of adenovirus E1A proteins. The sequences of the indicated adenovirus E1A proteins were aligned and labeled as described in the legend to Fig. 2. The zinc-finger, auxiliary region 1 (AR1), the binding site for CtBP, the canonical monopartite nuclear localization sequence (NLS), and the conserved phosphorylation sites are indicated.

Conservation of functional motifs and binding sites between E1A types

We examined whether known binding sites for E1A interacting proteins, sites of covalent modification, and well-characterized functional motifs were conserved among our expanded collection of HAdV and SAdV E1A proteins.

Rb interaction sites

HAdV-5 E1A makes two separate contacts with Rb (Dyson et al., 1992). The major interaction requires the

sequence LXCXE, where *X* is variable, present within CR2 of E1A (Dyson et al., 1989). This motif is absolutely conserved in all the E1A sequences (Fig. 2, a.p. 150, 152, and 154), underscoring the importance of this interaction for viral activation of cell cycle progression and transcription (Bayley and Mymryk, 1994). In all the E1A proteins, an invariant aspartic acid residue precedes this consensus site, suggesting that they all bind Rb with high affinity (Heck et al., 1992).

The crystal structure of the relevant portion of Rb with a peptide corresponding to the orthologous LXCXE sequence from the human papillomavirus 16 E7 protein indicates that each key residue of the motif fits tightly, making a high density of contacts in a shallow groove on the surface of Rb that ensures specificity (Lee et al., 1998). An additional leucine positioned two residues after the LXCXE motif in E7 interacts with a hydrophobic pocket on Rb, but the broader nature of this pocket likely allows some flexibility in the identity and position of this residue. Based on this structure, it was suggested that the corresponding contact in HAdV-5 E1A is the phenylalanine at position 129 (a.p. 157), which is located three residues after the motif. All the E1A proteins contain a hydrophobic residue at this position, suggesting that this somewhat less specific contact with Rb is maintained. The crystal structure also suggests that a patch of six basic residues on the rim of the LXCXE-binding groove in Rb could interact with the acidic residues present in E7 after the LXCXE motif, although these were not present in the E7 peptide used for the crystal structure (Lee et al., 1998). It was later confirmed that conversion of these residues in Rb from basic to acidic reduces or abolishes interaction of E7 or SV40 large T antigen, which also contains an LXCXE motif followed by a string of acidic residues (Brown and Gallie, 2002; Dick and Dyson, 2002). E7 binding to such an Rb mutant was restored by converting the acid residues following the LXCXE motif in E7 to basic residues, further demonstrating the necessity for this ionic interaction (Dick and Dyson, 2002). Like in E7 and large T antigen, a similar string of acidic residues following the LXCXE motif is present in all the different E1A proteins (Fig. 2, a.p. 161–169). However, mutational analysis of HAdV-5 and HAdV-12 E1A has not consistently shown a requirement for these residues for Rb binding (Alevizopoulos et al., 2000; Barbeau et al., 1994; Putzer et al., 1997).

In addition to mutations in the LXCXE motif, deletions within residues 30–60 were shown to impair the ability of HAdV-5 E1A to co-immunoprecipitate Rb (Egan et al., 1988; Wang et al., 1991; Whyte et al., 1989). Mutations in this region also blocked the ability of HAdV-5 E1A to dissociate Rb from E2F transcription factors (Raychaudhuri et al., 1991). This led to the identification of a second region of HAdV-5 E1A, spanning residues 37–54 (a.p. 44–63), which can independently interact with Rb, but with a greatly lower affinity (Dyson et al., 1992). This second Rb-binding site of E1A competes with E2F for interaction with Rb, and in combination with CR2 disrupts Rb–E2F complexes (Ikeda and Nevins, 1993). Indeed, deletion of residues 38–44 of HAdV-5 E1A, which comprise the N-terminal edge of CR1, abrogates interaction with Rb (Wong and Ziff, 1994). Interestingly, while the tyrosine at position 47 (a.p. 55) is also important for interaction with Rb, the neighboring highly conserved leucine at position 49 (a.p. 57) is not (Wang et al., 1993a). Taken together, these studies suggest that the interaction domain maps mainly between residues 37 and 47 of HAdV-5 E1A (a.p. 44–55), although additional residues are likely required as deletion of residues 48–60 (a.p. 56–69) also impairs binding (Barbeau et al., 1994; Egan et al., 1988). As currently defined, the second Rb interaction site overlaps the N-terminal boundary of CR1, which is absolutely conserved in all the E1A proteins with the exception of HAdV-41 (Fig. 2). Given the high degree of conservation of this region, its ability to mediate an interaction with Rb is probably a common feature of all E1A proteins. However, it is intriguing that mutation of the highly conserved leucine 49 (a.p. 57) to proline does not affect interaction with Rb, nor any other known function of HAdV-5 E1A (Wang et al., 1993a). Thus, the reason for its conservation, and refinement of the determinants for Rb interaction remain promising areas of investigation.

p300–CBP interaction sites

Similarly to its interaction with Rb, HAdV-5 E1A makes several distinct contacts with portions of the transcriptional co-activators CREB-binding protein (CBP) and the related p300 protein (Arany et al., 1995; Eckner et al., 1994; Kurokawa et al., 1998), as does HAdV-12 E1A (Fax et al., 2000; Lipinski et al., 1999). The $FX^{E}/_{D}XXXL$ consensus sequence located within CR1 of HAdV-2, -5, and -12 E1A interacts with the TRAM motif of CBP, located within the CH3 region (O'Connor et al., 1999). Small deletions within this region of HAdV-5 E1A block co-precipitation of p300/CBP (Barbeau et al., 1994; Mymryk et al., 1992; Wong and Ziff, 1994). Similarly, substitution of an alanine, but not aspartic acid, for the glutamic acid at the third position of the motif blocks the ability of HAdV-12 E1A to bind p300/CBP (Sawada et al., 1997). With the exception of HAdV-41, the key positions within this motif are well conserved in all sequences (Fig. 2, a.p. 75, 77, 81). This is in agreement with our observation that representative E1A proteins from all six subgroups bind CBP (Shuen et al., 2003). In addition, mutation of the arginine at position 2 to glycine or the histidine at position 3 to asparagine disrupts or seriously reduces the ability of HAdV-5 E1A to co-precipitate full-length p300/CBP from human HeLa cells (Wang et al., 1993a; Whyte et al., 1989). Mutation of the histidine at position 3 or deletion of residues 6–10 in HAdV-5 E1A (a.p. 9–14) also specifically reduces interaction with the CH3 region of CBP or p300 (Boyd et al., 2002; Kurokawa et al., 1998). These results suggest that the extreme N-terminal portion of HAdV-5 E1A functions in concert with the TRAM-binding region described above to contact the CH3 region of p300/CBP. While the arginine at position 2 is

absolutely identical in all the different E1A sequences, the histidine at position 3 is not. This suggests that interaction between the N-terminal portions of the different E1A proteins and the CH3 region of p300/CBP are not precisely the same. This is substantiated by the observation that the arginine at position 2 in HAdV-12 E1A is not required for binding to p300, although it is in HAdV-5 E1A (Lipinski et al., 1999).

In addition to interacting with the CH3 region of p300/CBP, the HAdV-5 and -12 E1A proteins bind to the C-terminal portion of CBP spanning residues 2067–2112 (Kurokawa et al., 1998; Lin et al., 2001; Lipinski et al., 1999). This region of CBP is also bound by several cellular transcription factors, including IRF-3, Ets-2, p53, and members of the NCoA/p160 family, and HAdV-5 E1A can compete with at least some of these for this interaction (Kamei et al., 1996; Lin et al., 2001; Livengood et al., 2002; Matsuda et al., 2004). The extreme N-terminus of HAdV-5 E1A is not required for this interaction, as mutation of the histidine at position 3 does not affect binding (Kurokawa et al., 1998; Lin et al., 2001). The solution structure of this region of CBP complexed with the AD1 region of ACTR, an NCoA/p160 protein, shows that these domains undergo mutual synergistic folding to form a helical heterodimer (Demarest et al., 2002). Sequence alignment of E1A with ACTR and other cellular proteins that bind this region of CBP demonstrates that they all possess limited sequence homology, largely composed of a putative amphipathic α-helix (Matsuda et al., 2004). In HAdV-5 E1A, this region spans residues 19–28 (LLDQLIEEVL, a.p. 23–32) and is located within the relatively non-conserved N-terminus. Our analysis indicates that the second leucine in this motif is absolutely conserved in all the different E1A proteins. Mutation of this leucine, but not the adjacent aspartic acid to alanine, in HAdV-5 E1A greatly reduces its ability to co-precipitate full-length p300/CBP from human HeLa cells, further supporting a role for this region in interaction with p300/CBP (Wang et al., 1993a). In addition, deletion of residues 20–24 of HAdV-5 E1A (a.p. 24–28) abolishes co-precipitation of p300/CBP (Wong and Ziff, 1994).

Sequences closely resembling this putative α-helix consensus motif are present in all the subgroup B and C E1A proteins (Fig. 2). Ets-2 and the NCoA/p160 protein SRC-1 contain phenylalanine or tryptophan at key positions in this CBP interaction motif, suggesting that these residues may functionally substitute for leucine in this interaction (Matsuda et al., 2004). Intriguingly, variants of this motif containing phenylalanine in place of leucine are usually present in subgroup A and D proteins and more divergent sequences resembling this motif are present closer to the N-terminus of SAdV-7, HAdV-40, and 41. Phenylalanine at the terminal position of this motif likely maintains interaction, as deletion of residues 26–35 in HAdV-5 E1A (a.p. 30–39), which removes the C-terminal portion of the motif, but brings phenylalanine 38 (a.p. 45) into place, still efficiently co-precipitates p300/CBP (Barbeau et al., 1994; Egan et al., 1988). Overall, the consensus motif in the various E1A proteins is ΨΨXXΨΨXXΨΨ (where Ψ is generally I, L, V, M, or F). It seems highly likely that even divergent variants of this motif, such as that found in HAdV-12 E1A (ILEHLVDNFF), confer functional interaction with the C-terminal portion of p300/CBP as mutation of the first two residues of this motif in HAdV-12 E1A severely reduces binding to p300/CBP (Fax et al., 2000; Lipinski et al., 1999). However, additional mutational studies will be necessary to confirm a role for this region in the interaction of the different E1A proteins with the C-terminal portion of CBP.

CtBP interaction site

The interaction of the transcriptional co-repressor CtBP with HAdV-5 and HAdV-12 E1A requires the sequence PLDLS near the C-terminus within CR4 (Molloy et al., 1998; Schaeper et al., 1995). Deletion of residues 239–254 reduces the interaction of HAdV-5 E1A with CtBP (Boyd et al., 1993; Schaeper et al., 1995; Zhang et al., 2001), and these sequences correspond to the N-terminal portion of CR4 (a.p. 295–311). The role of the remaining portion of CR4 remains unknown.

The PLDLS motif or homologous variants are present in all E1A types with the exception of the proteins of SAdV-21 and the subgroup E viruses, which contain the variant PLDLC. Intriguingly, this substitution was isolated as a naturally occurring mutation in the PLDLS motif of human homeodomain protein TGIF, which results in a developmental defect called holoprosencephaly (Gripp et al., 2000). In TGIF, this mutation blocks interaction with CtBP and impairs transcriptional repression (Melhuish and Wotton, 2000). Thus, CtBP is probably not targeted by the E1A proteins of SAdV-21 or the subgroup E viruses, most of which are simian in origin as well. This differential requirement for CtBP interaction will likely prove an interesting area of future investigation.

An adjacent lysine residue is present within one or two residues of the CtBP-binding motif in all thirty-four E1A sequences. Acetylation of this lysine in HAdV-5 E1A by p300 or pCAF may block interaction with CtBP (Zhang et al., 2000), but this has not been confirmed (Madison et al., 2002).

BS69 interaction site

The transcriptional co-repressor and potential tumor suppressor BS69 has been demonstrated to bind the PXLXP motif present in cellular and viral proteins including E1A (Ansieau and Leutz, 2002). This sequence is located immediately upstream of CR2 in HAdV-5 E1A, but appears to be weakly conserved across the various adenovirus species. It remains to be determined whether this interaction is specific to a subset of E1A proteins. The CR3 region of HAdV-5 E1A also independently binds BS69 (Hateboer et al., 1995). The consequences of the E1A–BS69 interaction remain to be elucidated, although overexpression of BS69

can inhibit transcriptional activation by CR3 (Hateboer et al., 1995).

TBP interaction sites

As seen with Rb or p300/CBP, two separate regions of E1A contact the TBP component of the general transcription factor IID. The N-terminal portions of HAdV-5 and -12 E1A each interact with TBP in vitro, and this binding maps within the first 29 residues of each protein (Lipinski et al., 1998; Song et al., 1995, 1997). Detailed mapping of the interaction of HAdV-5 E1A with TBP indicates that the most critical contact is the cysteine at position 6 (a.p. 9) (Boyd et al., 2002), which is present only in the subgroup C E1A proteins. Despite the absence of a cysteine in a comparable position in the N-terminal portion of HAdV-12 E1A, the first 29 residues of HAdV-12 E1A bind TBP (Lipinski et al., 1998). It is thought that the interaction of the N-terminal portion of HAdV-5 E1A with TBP contributes to transcriptional repression by dissociating TBP from the TATA box (Boyd et al., 2002; Song et al., 1995, 1997). However, additional studies will be required to define the contacts before any generalizations can be made regarding the potential ability of the other E1A proteins to perform this activity.

In addition to the N-terminal interaction site, CR3 of HAdV-5 E1A independently targets TBP as part of the mechanism by which it activates transcription (Boyer and Berk, 1993; Lee et al., 1991). Binding has been mapped to specific residues within the zinc-finger portion of CR3 in exquisite detail (Geisberg et al., 1994) and will be discussed further in the transcriptional activation section below.

Proteasome interaction sites

Both the HAdV-5 and HAdV-12 E1A proteins have been shown to interact with the Sug1 (S8) ATPase component of the 19S regulatory complex of the proteasome (Grand et al., 1999). This interaction requires a region in HAdV-5 E1A spanning residues 4–25 (a.p. 4–29), which also makes an independent contact with the S4 ATPase of the 19S regulatory complex (Turnell et al., 2000). Based on the low level of homology between this region of HAdV-5 and -12, it is entirely possible that this interaction is maintained in the other less characterized E1A proteins. An additional interaction with the proteasome is mediated by the region spanning residues 111–127 of HAdV-5 E1A (a.p. 137–155), which overlaps the N-terminal boundary of CR2 and contacts the non-ATPase S2 component of the 19S regulatory complex (Zhang et al., 2004). Given the high level of sequence conservation in this region, it seem likely that S2 represents a common target of the E1A family of proteins.

Nuclear import signals

HAdV-5 contains an NLS at its carboxyl terminus, composed of the sequence KRPRP (Lyons et al., 1987), which is preferentially recognized by importin-α3 (Kohler et al., 2001) and does not function in growth-arrested cells (Lyons, 1991). This motif is representative of a canonical monopartite NLS, which is typified by the presence of four adjacent basic residues, or three basic residues in combination with proline (Macara, 2001). With the exception of SAdV-7 and -21 and the subgroup B:2, all E1A proteins contain predicted canonical monopartite NLS near their C-termini (Fig. 3, a.p. 350–355), suggesting that nuclear localization via the carboxyl end is a property common to most E1As. Whether these are preferentially targeted by importin-α3 remains to be determined. Acetylation of lysine 285 within the C-terminal NLS of HAdV-5 E1A (a.p. 350) by CBP blocks interaction with importin-α3 and is associated with retention of the smaller 243R E1A protein in the cytoplasm (Madison et al., 2002). Thus, subcellular localization of the HAdV-5 E1A 243R protein may be regulated by covalent modification. By extension, a similar regulatory mechanism may exist for the smaller E1A proteins of other serotypes. In addition to the C-terminal NLS, a non-conventional NLS with the consensus sequence $FV(X)_{7–20}MXSLXYM(X)_4MF$ has been identified within CR3 of HAdV-5, which is unique to the larger 289R protein (Slavicek et al., 1989; Standiford and Richter, 1992). This signal functions in a developmentally regulated fashion (Standiford and Richter, 1992) and its presence in the 289R E1A protein can confer nuclear localization irrespective of the acetylation state of the C-terminal NLS (Madison et al., 2002). Some of the key residues of the CR3 NLS are not well conserved in other E1A proteins (Fig. 3, a.p. 197, 198, 225, 227, 228, 230, 231, 236, and 237), suggesting that this function may be unique to certain adenoviral species.

Phosphorylation sites

Both HAdV-5 and HAdV-12 E1A proteins are extensively phosphorylated at multiple serines mapped to positions 89, 96, 132, 185, 188, and 219 in HAdV-5 E1A (Dumont and Branton, 1992; Dumont et al., 1989, 1993; Lucher et al., 1985; Richter et al., 1988; Smith et al., 1989; Tremblay et al., 1989; Tsukamoto et al., 1986; Whalen et al., 1997). Only serines 132, 185, and 188 (a.p. 160, 240 and 243) are conserved, and they are absolutely invariant in all the E1A sequences. Interestingly, these are also the only three phosphorylation sites within HAdV-5 E1A that are known to affect its biological function. For example, phosphorylation of HAdV-5 E1A serine 132 (a.p. 160), which is adjacent to the LXCXE motif, enhances binding to Rb (Whalen et al., 1996), and may increase its ability to disrupt Rb–E2F complexes (Mal et al., 1996a). In all the different E1A proteins, this site also conforms to a consensus casein kinase II site (S/TXXD/E), and in HAdV-5, this site is phosphorylated by this kinase in vitro (Whalen et al., 1996). Similarly, phosphorylation of serines 185 and 188 (a.p. 240 and 243) in HAdV-5 E1A specifically increases transcription of the E4 gene (Whalen et al., 1997). Given the absolute conservation of these residues and their adjacent sequence, it seems likely that phosphorylation of

these serines in the other E1A proteins will have similar effects.

Transcriptional activation by E1A

HAdV-5 E1A strongly activates transcription of viral and cellular genes (Bayley and Mymryk, 1994; Berk et al., 1979; Brockmann and Esche, 2003; Jones and Shenk, 1979; Shenk and Flint, 1991). Two major activities reside within the N-terminal/CR1 and CR3 regions of E1A (Akusjarvi, 1993; Bayley and Mymryk, 1994; Brockmann and Esche, 2003; Shenk and Flint, 1991). It is currently thought that recruitment of these transcriptional activation surfaces of E1A to target genes occurs through interaction with sequence specific DNA-binding factors, as HAdV-5 E1A does not bind DNA specifically (Avvakumov et al., 2002a; Chatterjee et al., 1988; Zu et al., 1992).

When fused to the Gal4p DBD, a transcriptional activation region in HAdV-5 E1A has been identified that overlaps CR1, located within residues 28–82 (a.p. 32–91) (Bondesson et al., 1994; Shuen et al., 2002). A similar activity also resides within CR1 of HAdV-12 E1A (Lipinski et al., 1997). The strong conservation of sequence within CR1 suggests that all E1A proteins contain a transcriptional activation surface in this region. In support of this, we have shown that the N-terminal/CR1 portions of HAdV-3, 4, 9, 12, and 40 all interact with the transcriptional co-activators CBP, p300, and pCAF (Shuen et al., 2003), as previously shown for HAdV-5 E1A.

When fused to the Gal4p DBD, a second transcriptional activation domain of HAdV-5 E1A was identified within residues 139–223 (a.p. 168–279) (Lillie and Green, 1989), which encompass CR3. This region had already been known to be critical for activation of viral early gene expression (Glenn and Ricciardi, 1985; Jelsma et al., 1988; Lillie et al., 1986; Moran et al., 1986; Schneider et al., 1987; Svensson and Akusjarvi, 1984), but this observation provided a new approach for detailed study of the mechanism of transcriptional activation. Deletion analysis indicated that the N-terminal boundary of this transcriptional activation region maps to residues 141/142 (a.p. 195/197) (Martin et al., 1990). In HAdV-5 E1A, the C-terminal boundary was originally placed at residue 188 (a.p. 243), as a peptide corresponding only to residues 140–188 (a.p. 169–243) is sufficient to at least partially activate some viral early gene expression during infection with a virus lacking E1A (Lillie et al., 1987). Later work showed that two auxiliary regions (AR), named AR1 and 2, encoded within exon 2 of HAdV-5 E1A are interchangeably required for efficient transcriptional activation of the adenovirus E4 promoter (Bondesson et al., 1992). AR1 maps between residues 189 and 200, while AR2 maps between residues 224 and 245 in HAdV-5 E1A (a.p. 244–256 and 280–301, respectively). Further analysis demonstrated that AR2 can only substitute for AR1 when artificially juxtaposed to CR3 (Strom et al., 1998). The negative charge present within AR1, but not the exact protein sequence, is critically important for E1A mediated transcriptional activation of five different adenovirus early promoters, as well as that of cellular HSP70. This suggests that AR1 should be regarded as an integral part of the CR3 transactivation domain (Strom et al., 1998).

The alignment of the thirty-four E1A sequences demonstrates that CR3 is very highly conserved. This is expected given the critical role of this region in the activation of viral transcription (Bayley and Mymryk, 1994; Shenk and Flint, 1991). Although the six repeats of glutamic acid-proline found in AR1 of HAdV-5 E1A are not highly conserved, most of the other E1A proteins have several acidic residues within the corresponding region. In addition, many have a preponderance of serine or threonine residues, which could potentially contribute negative charge upon phosphorylation. This overall conservation of negative charge agrees with the demonstration that it is the acidic character of this region, rather than the exact sequence that is critical for transcriptional activation (Strom et al., 1998). Conversely, little conservation in the AR2 region is apparent, except for small isolated blocks of sequence. This lack of conservation coincides with the observation that this region is not normally required for transactivation (Strom et al., 1998).

Transcriptional activation by the CR3 region of HAdV-5 E1A has been analyzed extensively using site-directed mutagenesis (Fahnestock and Lewis, 1989; Lillie et al., 1986; Jelsma et al., 1988; Martin et al., 1990; Webster and Ricciardi, 1991). Indeed, every residue from 137 to 190 of HAdV-5 E1A (a.p. 166–245) has been individually mutated to at least one other amino acid. Studies of these mutants have identified the key residues necessary for function and produced a detailed understanding of the mechanism of transcriptional activation by CR3. We have taken advantage of this large body of data and used it to determine if the key residues within CR3 that are required for transcriptional activation by HAdV-5 E1A are conserved within the E1A proteins of the other virus types (Fig. 4). We calculated the frequency of amino acid conservation at each position in the CR3 alignment of all non-subgroup C E1A proteins with respect to HAdV-5 E1A. This was compared to residues critical for transcriptional activation by HAdV-5 E1A, as identified by point-mutation studies (Lillie et al., 1986; Jelsma et al., 1988; Martin et al., 1990; Webster and Ricciardi, 1991). With only two notable exceptions, all residues identified as essential for transcriptional activation are conserved in the majority of E1A proteins. In the first exception, the conservative substitution of leucine for valine 147 in HAdV-5 E1A (a.p. 202) abolishes transcriptional activation (Webster and Ricciardi, 1991). However, the vast majority of other E1A proteins contain a proline at this position. The presence of proline at this position is likely tolerated as the CR3 portion of HAdV-12 E1A strongly activates early viral transcription (Ohshima and Shiroki, 1986). Structural analysis and peptide competition experiments also fail to explain why substitution of leucine for valine at position 147 was observed to have such a

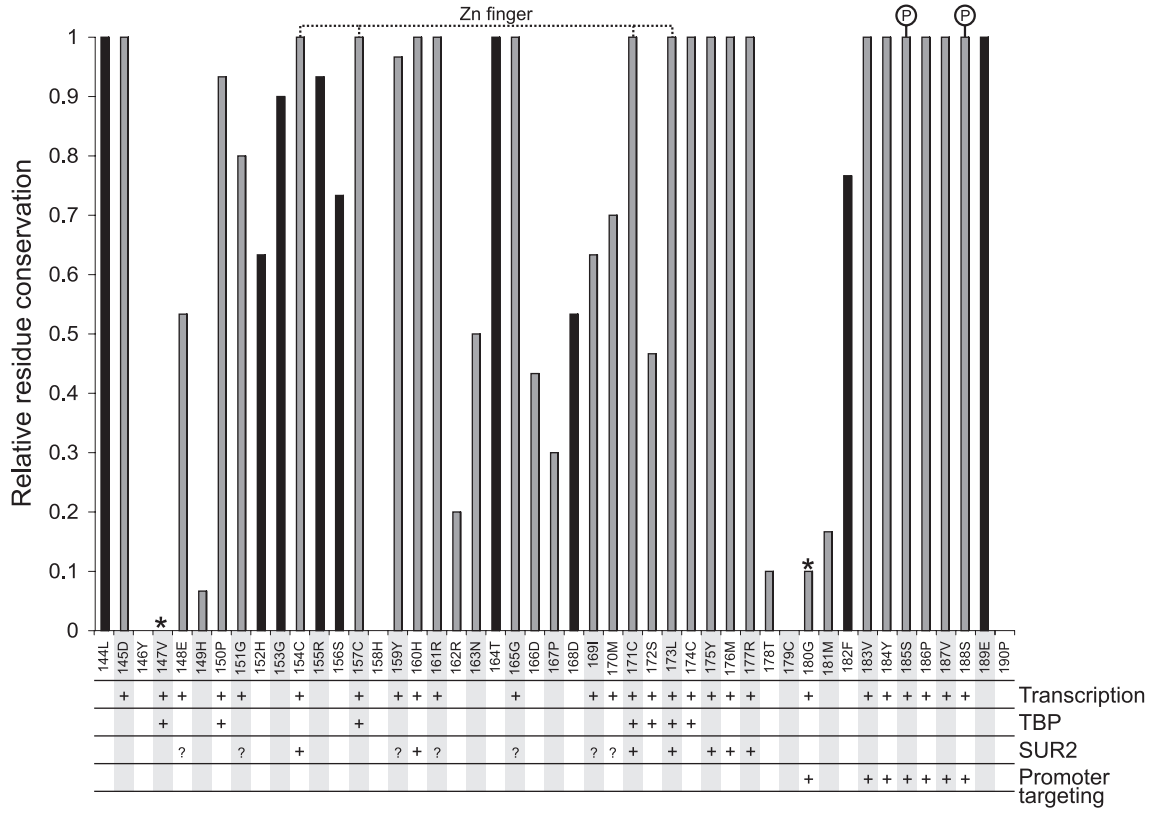

Fig. 4. Analysis of functional conservation of residues within CR3 required for transcriptional activation. The percent identity of each adenoviral E1A sequence to that of HAdV-5 was calculated for each position corresponding to residues 144 to 190 in HAdV-5 (alignment positions 199 to 245). HAdV-1, 2, and 6 E1A were excluded as they are identical to HAdV-5 over this region. Residues previously identified by point-mutational analysis as critical for HAdV-5 E1A transcriptional activation, interaction with TBP, interaction with Sur2 or promoter targeting are indicated as "+". Point mutants at each position have been tested for all functions with the exception of Sur2 interaction. Question marks indicate residues that are essential for transcriptional activation, but their involvement in TBP or Sur2 interaction is not clear. Asterisks indicate residues identified as critical for activity in HAdV-5 E1A that are not highly conserved. Black bars indicate residues not known to be required for transcriptional activation, but for which only conservative substitutions have been tested that are often present at the same alignment positions in the other E1A proteins. Hatched bars indicate highly conserved residues (>60% similarity) not required for transcriptional activation. The position of the zinc finger and the two phosphorylated serines in HAdV-5 E1A are indicated.

pronounced impact on transcription (Molloy et al., 1999). In the second exception, substitution of an aspartic acid or cysteine for glycine 180 (a.p. 235) seriously impairs transactivation by HAdV-5 E1A (Webster and Ricciardi, 1991), but only the HAdV-40 and -41 E1A proteins contain glycine at this position. Notably, all the subgroup B HAdV E1A proteins contain a cysteine at this position, indicating that they may be poor transcriptional activators. Substitution of alanine or asparagine at position 180 in HAdV-5 E1A retains function, suggesting that there is some flexibility in composition at this location. Indeed, six of the other non-subgroup C E1A proteins contain asparagines at this position.

Our analysis also shows that many residues not identified as important for transcriptional activation by mutational analysis are very highly conserved. For residues 144, 155, 156, 164, 168, 182, and 189 (a.p. 199, 210, 211, 219, 223, 237, 244; indicated as black bars in Fig. 4), only conservative substitutions have been tested that are often present at the same alignment positions in the other E1A proteins. We would predict that non-conservative substitutions at these positions will impair transcriptional activation. On the other hand, residues 152 and 153 (a.p. 207 and 208; hatched bars in Fig. 4) are conserved in more than 60% of the E1A sequences, although they have been conclusively shown to be dispensable for transcriptional activation. This suggests that they either play a role in another activity of E1A or only influence transcriptional activation under specific circumstances not yet tested in the laboratory.

CR3 can be subdivided into at least three functional domains: a promoter targeting region, a TBP interaction region and a Sur2/TRAP150β-binding region. In HAdV-5 E1A, residues 180–188 (a.p. 235–243) constitute a promoter targeting region, which confers interaction with several unrelated sequence specific transcription factors, including ATF1-3, c-jun, SP1, USF, Oct-4, and CBF/NF-Y (Agoff and Wu, 1994; Chatton et al., 1993; Liu and Green, 1990, 1994; Scholer et al., 1991). It is difficult to imagine how this short region of E1A makes specific contacts with such a diverse range of unrelated transcription factors. Indeed, this region of E1A also interacts with several TBP associated factors (TAFs), including $TAF_{II}250$, $TAF_{II}135$, $TAF_{II}110$, and $TAF_{II}55$ (Chiang and Roeder, 1995; Geisberg et al., 1995;

Mazzarelli et al., 1995, 1997). TAFs function as promoter selective transcriptional regulators via mechanisms that are not yet fully defined. However, many are known to interact with transcription factor activation domains, and all function as components of multi-subunit protein complexes (Green, 2000). This raises the possibility that the promoter targeting region of HAdV-5 E1A CR3 binds a single TAF directly, which then indirectly mediates interaction with other TAFs and specific classes of transcription factors. Regardless of whether HAdV-5 E1A is recruited directly or indirectly by specific DNA-binding transcription factors, this targeting is normally necessary for transcriptional activation. However, this region is not required for transcription if HAdV-5 E1A is artificially tethered to a promoter by fusion to a heterologous DNA-binding domain (Lillie and Green, 1989; Martin et al., 1990). Our analysis shows that the carboxy-terminal portion of the promoter targeting region is highly conserved in all the E1A proteins, indicating that they each likely target the same cellular transcription factors, be they DNA-binding proteins or TAFs.

Mutations in the CR3 promoter targeting region exhibit a characteristic dominant negative effect on transcriptional activation by wild-type HAdV-5 E1A, presumably by sequestering limiting factors necessary for this activity (Glenn and Ricciardi, 1987; Webster and Ricciardi, 1991). The first of these to be identified was TBP (Lee et al., 1991). Detailed analysis of the interaction of HAdV-5 E1A CR3 with TBP localized this interaction to residues 147, 150, 157, and 171 to 174 (a.p. 202, 205, 212 and 226–229), which are in or around the zinc finger region (Geisberg et al., 1994). With two notable exceptions, these residues are well conserved. Firstly, the valine at position 147 (a.p. 202) is only conserved in the subgroup C E1A proteins as described above. Despite a proline at this position, which is present in many of the other E1A proteins, HAdV-12 E1A binds TBP very strongly in a CR3-dependent fashion (Molloy et al., 1999). In the second exception, the E1A proteins from subgroups D, E, and F contain a substitution of alanine for serine at position 172 (a.p. 227), which suggests that they will bind TBP poorly. It will be interesting to compare the transcriptional activities of these proteins and to determine the consequences of decreased, or possibly increased, activity.

Although a clear relationship exists between the ability of HAdV-5 E1A to bind TBP and activate transcription, mutation of residues 148, 151, 154, 159, 161, 165, 169, 170 or 177 (a.p. 203, 206, 209, 214, 216, 220, 224, 225 and 232) blocks transcriptional activation despite retaining TBP interaction (Geisberg et al., 1994). This observation indicated that at least one other factor is essential for transactivation by E1A, and ultimately led to the identification of the Sur2/TRAP150β component of the Mediator–TRAP complex as a target of the CR3 domain of HAdV-5 E1A (Boyer et al., 1999; Wang and Berk, 2002). Sur2 forms a subcomplex of the Mediator with Med/TRAP100 and 95, and cells deficient in either Sur2 or Med/TRAP100 do not support transcriptional activation by HAdV-5 E1A (Ito et al., 2002; Stevens et al., 2002). Mutation of positions 154, 160, 171, 173, 175, 176, or 177 (a.p. 209, 215, 226, 228, 230, 231, and 232) blocks association with Sur2 (Boyer et al., 1999), and these are all highly conserved residues in the different E1A proteins. Many of the mutants that fail to bind Sur2 retain TBP binding, but fail to activate transcription, indicating that Sur2 is one of the previously unknown key targets of CR3. However, a class of mutants represented by positions 148, 151, 159, 161, 165, 169, and 170 (a.p. 203, 206, 214, 216, 220, 224, and 225) in HAdV-5 E1A are defective for transcriptional activation (Martin et al., 1990) and remain untested for interaction with Sur2 or TBP (Fig. 4). Thus, there remains the exciting possibility that these residues constitute a binding surface for yet another key factor required for transcriptional activation.

Identification of highly conserved residues without known function

As mentioned above, examination of the sequence alignment indicates that a substantial number of highly conserved amino acid residues within CR3 are required for E1A function yet are not known to affect interaction with any cellular protein. In addition, two residues within CR3 have been conclusively shown not to affect function, yet remain highly conserved (Fig. 4). Close inspection reveals that a similar situation exists throughout the entire E1A alignment. Indeed, several absolutely conserved residues, particularly within CR4, have neither known function nor cellular target. Highly conserved residues that are not known to be involved in any E1A function or protein interaction are indicated in Figs. 2 and 3. They may contribute to known protein interactions or functions of E1A, or may represent key determinants of novel functions that remain to be elucidated. Either possibility opens new avenues of future investigation.

Conclusions

The analyses presented here strengthen and refine the positioning of the conserved regions within E1A. A number of protein interaction motifs are unambiguously present in many E1A species, suggesting a strong selective pressure to maintain certain protein–protein interactions. Despite the overall commonality of many of the interaction motifs, our analysis reveals that clear differences exist between many serotypes and the prototype HAdV-5 E1A. Exploration of these differences will greatly expand our understanding of the normal biological functions of E1A and assist in the use of E1A as a tool to probe cellular processes. In particular, this sequence alignment should aid in the identification and precise definition of interaction surfaces of E1A required for targeting cellular proteins.

Importantly, our analysis demonstrates that highly conserved subregions throughout the E1A proteins still do not have identified functions. Discovery of their targets and function remain promising areas of investigation. Furthermore, the detailed understanding of the structure–function relationship in E1A is already being utilized in the development of conditionally replicating HAdV-5 as oncolytic agents (Fueyo et al., 2000, 2003; Gomez-Manzano et al., 2004; Heise et al., 2000; Howe et al., 2000). Our analysis will similarly allow exploitation of other serotypes with altered specificity of infection or to which pre-existing immunity is rare.

Materials and methods

Provenance and propagation of virus

Viruses were obtained from the American Type Culture Collection (Manassas, VA, USA), Dr. D. Erdman (Center for Disease Control and Prevention, Atlanta, GA, USA) or the Leiden University Medical Centre, Department of Molecular Cell Biology virus collection (Leiden, The Netherlands) as detailed in Table 1. Viruses were propagated on monolayer cultures of A549 human lung epithelial carcinoma cells.

Sequence determination and manipulation

Viral sequences were determined as described previously (Avvakumov et al., 2002b). Briefly, mRNA was isolated from A549 cells 6 h post-infection using Trizol (Invitrogen, Burlington, ON, Canada) and used as a template for Moloney murine leukemia virus reverse transcriptase (Invitrogen) to generate total cDNA. The E1A gene was then amplified with subgroup-specific primers designed to anneal within highly conserved regions upstream and downstream of the E1A coding region. PCR products were subcloned into suitable vectors, and the E1A gene was sequenced with vector-specific flanking primers. The newly determined E1A sequences were deposited in GenBank and their accession numbers are listed in Table 1.

Additional E1A sequences were obtained from GenBank and are also listed in Table 1. Alignment and analysis of the largest predicted E1A proteins were performed as before (Avvakumov et al., 2002b), using CLUSTAL W (Thompson et al., 1994) with default parameters except that the gap open cost was set to 2. Alignments were edited manually using GeneDoc (Nicholas et al., 1997) and shaded to four levels of conservation using the blosum 45 score table. The final alignment was imported into the PHYLIP suite of software to calculate phylogenetic distance using the programs PROTDIST.EXE and NEIGHBOR.EXE (Dr. Joe Felsenstein, University of Washington, Seattle, WA, USA) and visualized with TreeView (Page, 1996).

Definition of conserved regions (CR)

To define the boundaries of the CRs precisely, a stringent cutoff for each edge was adopted based on 100% identity or similarity, with nine or fewer consecutive less conserved residues occurring between absolutely conserved residues. The average CR4 similarity between HAdV-5 E1A and all other sequences, with the exception of the closely related HAdV-1, 2, and 6, was calculated manually.

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